The C2 Domain of the Ubiquitin Protein Ligase Nedd4 Mediates Ca²⁺-dependent Plasma Membrane Localization*

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Neuronal precursor cell-expressed developmentally down-regulated 4 (Nedd4) is a ubiquitin protein ligase (E3) containing a hect domain, 3 or 4 WW domains, and a putative C2 domain. We have recently demonstrated an association between the WW domains of Nedd4 and the proline-rich PY motifs (XPPXY) of the epithelial Na⁺ channel, as well as with PY motifs of several other proteins. The role of the putative C2 domain of Nedd4 has not been elucidated. Here we show that Nedd4, endogenously expressed in Madin-Darby canine kidney cells, was redistributed from the cytosolic to the particulate fraction in response to ionomycin plus Ca²⁺ treatment. A similar treatment of polarized Madin-Darby canine kidney cells led to an apical and lateral membrane localization of Nedd4, as determined by immunostaining and confocal microscopy. The C2 domain of Nedd4, expressed as a glutathione S-transferase (GST) fusion protein, was sufficient to bind cellular membranes in a Ca²⁺-dependent manner. Moreover, this GST-Nedd4-C2 domain was able to mediate Ca²⁺-dependent interactions with phosphatidylserine, phosphatidylinositol, and phosphatidylcholine liposomes *in vitro*. An epitope-tagged Nedd4 lacking its C2 domain and stably expressed in Madin-Darby canine kidney cells failed to mediate the Ca²⁺-induced plasma membrane localization seen in wild-type (epitope-tagged) Nedd4. These results indicate that the putative C2 domain of Nedd4 acts as a bona fide C2 domain which binds phospholipids and membranes in a Ca²⁺-dependent fashion and is involved in localizing the protein primarily to the apical region of polarized epithelial cells in response to Ca²⁺.

Neuronal precursor cell-expressed developmentally down-regulated 4 (Nedd4)[1] (1) is a multimodular ubiquitin protein ligase (E3) composed of a putative C2 domain, 3 (or 4) WW domains, and a C-terminal ubiquitin protein ligase hect domain (Fig. 1A). We have previously identified Nedd4 as a protein that interacts with the epithelial Na⁺ channel (ENaC); this interaction is mediated by an association of the WW domains of Nedd4 with the proline-rich PY motifs (XPPXY, where X = any amino acid) of the αβγ ENaC subunits (2). Deletion or mutations within the PY motifs of the β and γ subunits have been genetically linked to Liddle syndrome (3–6), a hereditary form of systemic renal hypertension caused by an abnormal increase in ENaC activity (7, 8). More recently, our own work and that of others (9) have described interaction of Nedd4 and Nedd4-like proteins with other PY motif-containing proteins, also mediated by the WW domains. We therefore speculate that substrate specificity for the E3 activity of Nedd4 is conferred, at least in part, by its WW domains (10). Unlike the WW domains, however, the function of the putative C2 domain of Nedd4 has not been elucidated.

The C2 domain is a module present in numerous proteins (11, 12), including protein kinase C (PKC) (13), p120^{ras}-GTPase activating protein (GAP) (14), the isoforms of phosphoinositide-specific phospholipase C (phospholipase C-β,γ,δ) (15–17), cytosolic phospholipase A₂ (18), and synaptotagmin (19) and has been shown to be a functional domain in several of these proteins (18, 20–24). The C2 domains described to date show significant diversity in the molecules they interact with, which include Ca²⁺, phospholipids, intracellular proteins, and inositol polyphosphates. The C2 domain spans approximately 120 amino acids and includes a region of conserved amino acids, the CaLB (Ca²⁺ lipid-binding) domain (18). Based on the three-dimensional structure of the synaptotagmin first C2 domain (25, 26) and the C2 domain of phospholipase Cδ (27), it has been proposed that the interaction between the domain and its Ca²⁺ ligand occurs via a "C2 key." This key consists of a ~70-residue core (including the CaLB region) that folds into a four-stranded β sheet, forming a cavity lined with five aspartate (or in some cases asparagine or glutamate) residues, the Ca²⁺-binding sites (Fig. 1B) (25–27). Accordingly, two models of the Ca²⁺-stimulated phospholipid binding by the C2 domain have been proposed. The first model suggests conformational changes upon Ca²⁺ binding which expose either hydrophobic residues to insert into the lipid bilayer or charged residues to associate with specific phospholipid headgroups (28). The other model proposes displacement of a Ca²⁺ coordinating water molecule with the headgroup or phosphate oxygens of phospholipid, providing direct Ca²⁺ coordination (for review, see Ref. 12).

Although the majority of C2 domains characterized thus far have been shown to bind Ca²⁺ directly, several C2 domains are believed to act independent of a direct Ca²⁺ interaction. Syn-

[1] The abbreviations used are: Nedd4, neuronal precursor cell-expressed developmentally down-regulated 4; ENaC, epithelial Na⁺ channel; PKC, protein kinase C; GAP, p120^{ras}-GTPase activating protein; MDCK, Madin-Darby canine kidney; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

aptotagmin, the membrane-spanning Ca^{2+} sensor in synaptic vesicles, contains two tandem C2 domains (29). In several synaptotagmin isoforms (tagmin I, II, III, V, and VII) the first C2 domain (C2A) binds phospholipids in a Ca^{2+}-dependent manner (30–32), whereas the other C2 domain (C2B) binds to phospholipids irrespective of the presence of Ca^{2+} (33, 34). The binding of polyphosphoinositides to the second C2 domain has been shown to be a Ca^{2+}-independent process (34, 35), although recent work (36) suggests that Ca^{2+} ions switch the specificity of C2B binding from phosphatidylinositol-3,4,5-trisphosphate (bound at resting Ca^{2+} concentrations) to phosphatidylinositol-3,4-bisphosphate (bound at Ca^{2+} concentrations required for transmitter release in nerve terminals). As such, in addition to the Ca^{2+}-dependent regulatory role of the C2A domain, the C2B domain of synaptotagmin could act as a Ca^{2+} sensor in nerve terminals for regulated neurotransmitter release.

In addition to the C2-lipid interactions, accumulating evidence suggests that at least some C2 domains bind proteins as well. The synaptotagmin C2A and C2B domains bind several proteins (for review, see Ref. 37) including Ca^{2+}-dependent binding to syntaxin, a membrane protein in the presynaptic terminal, and AP-2, a hetero-oligomeric adapter complex in clathrin-coated pits (32, 38, 39). In addition, Ca^{2+}-induced translocated PKC interacts with several proteins from the particulate fraction that only bind in the presence of PKC activators (40). These proteins, receptors for activated protein kinase C (RACKs), are substrates of activated PKC (41) and peptides that mimic the binding site of PKC to RACKs (which contain the PKC-C2 domain) inhibit PKC translocation. Recombinant fragments of synaptotagmin containing the C2 region bind to RACKs and inhibit PKC translocation and association with these proteins (42). In addition, recent work (43) has demonstrated a direct interaction between annexin VII and the CaLB domain of p120^{GAP}, thereby furthering the notion that C2/CaLB domains interact with proteins as well.

In the present study we show that Nedd4 expressed endogenously in Madin-Darby canine kidney (MDCK) cells associates with membranes and localizes to the plasma membrane in a Ca^{2+}-dependent fashion, with a particular preference for the apical and lateral regions of these polarized epithelial cells. We also demonstrate that the Nedd4-C2 domain by itself is capable of mediating a Ca^{2+}-dependent cellular membrane and phospholipid binding and that Nedd4 lacking its C2 domain (heterologously expressed in MDCK cells) fails to associate with the plasma membrane in response to Ca^{2+}. These data suggest that the C2 domain may serve to localize Nedd4 to the apical and lateral regions of polarized epithelia.

EXPERIMENTAL PROCEDURES

Membrane Preparation—The epithelial MDCK cells, expressing endogenous Nedd4, were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C, 5% CO_2 atmosphere. For ionophore treatment, cells were serum-starved for 16 h, trypsinized, resuspended in serum-free medium, and harvested. Cells were then washed twice with Ca^{2+}-free medium (140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 20 mM glucose, and 20 mM HEPES) and then incubated in that medium containing also 1 mM EGTA, pH 7.3, for 5 min at 37 °C in the presence or absence of 1 μM ionomycin and the indicated amount of free Ca^{2+} (maintained with Ca^{2+}-EGTA buffers). The application of ionomycin in the presence of Ca^{2+} has been shown to induce increases in cytosolic calcium in MDCK cells (44, 45). The cells were then collected and washed twice with Ca^{2+}-free medium (for $-Ca^{2+}$ conditions) or $+Ca^{2+}$ medium (same as $-Ca^{2+}$ medium but containing 1.1 mM $CaCl_2$) and resuspended in $+Ca^{2+}$ medium with 10 μg/ml of both leupeptin and aprotinin and 1 mM phenylmethylsulfonyl fluoride. Cells were then homogenized, and nuclei and mitochondria were pelleted at 1000 $\times$ g (5 min, 4 °C). The supernatant was then spun at 100,000 $\times$ g for 30 min (4 °C), and the cytosolic (supernatant, S100) and pellet (particulate, P100) fractions were

collected for further analysis.

Immunoprecipitations—Particulate (P100) fractions were resuspended in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of both leupeptin and aprotinin). Nedd4 was immunoprecipitated from the S100 and P100 fractions using an affinity-purified polyclonal antibody raised against glutathione S-transferase (GST)-Nedd4-WWII (2) by incubating for 4 h at 4 °C. The immunocomplexes were collected with protein A-Sepharose beads, washed five times with 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol. Proteins were separated on 8% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Nedd4-WWII antibodies followed by horseradish peroxidase-conjugated secondary antibodies and ECL detection (Amersham Corp.).

Preparation of GST Fusion Protein Constructs—GST fusion proteins were prepared by polymerase chain reaction amplification of the region of rNedd4 cDNA (2) corresponding to the C2 domain (nucleotides 182–574). Polymerase chain reaction fragments were subcloned with flanking *Bam*HI and *Eco*RI sites into the corresponding sites in pGEX-2TK (Pharmacia Biotech Inc.). The plasmid containing the C2 insert, verified by sequencing, were used to transform the HB101 strain of *Escherichia coli*. Fusion protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (Pharmacia) for 4–5 h, and bacteria were collected and lysed by sonication in phosphate-buffered saline containing aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (concentrations as stated above). Fusion proteins were purified from the bacterial lysate with glutathione-agarose beads and eluted with 30 mM reduced glutathione (pH 8.0).

In Vitro Binding Experiments—For translocation assays, 200 μg (by protein determination) of crude membranes from MDCK cells, prepared as described above, were diluted into Ca^{2+}-free medium, pH 7.5, containing 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of both leupeptin and aprotinin. Membranes were then resuspended by sonication (5 $\times$ 1 s) and incubated with purified GST alone or GST-C2 (10 nM) in the presence or absence of Ca^{2+} for 30 min at 30 °C. Membrane-associated proteins were separated on 10% SDS-PAGE and immunoblotted with anti-GST antibodies as described above.

For liposome binding experiments, liposomes of varying composition (either mixtures of or pure phosphatidylserine, phosphatidylinositol, or phosphatidylcholine) were used. Approximately 1 mg of the phospholipid in chloroform was dried under nitrogen, added to 1 ml of sucrose-containing media (25 mM HEPES, 0.1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 20% sucrose, pH 7.5), and sonicated at 4 °C five times for 1 s to resuspend. One hundred microliters of liposomes were incubated with 10 nM purified GST-C2 or GST alone in the presence of Ca^{2+} (1 mM) in a final assay volume of 1 ml. Liposomes were collected by centrifugation at 100,000 $\times$ g for 30 min at 4 °C and proteins associated with them separated on 10% SDS-PAGE and immunoblotted with anti-GST antibodies, as described above.

Generation of Stable MDCK Cell Lines Expressing Epitope-tagged Nedd4 or C2-deleted Nedd4—A C2-deleted (ΔC2) Nedd4 was created by deleting amino acids 79–191 (nucleotides 179–571) of Nedd4 using polymerase chain reaction. This construct, as well as full-length Nedd4 used as a control, were epitope-tagged with the T7 epitope (MASMTG-GQQMG) placed at the N terminus of the molecule. These constructs, called T-Nedd4 or T-ΔC2-Nedd4, were subcloned into the pRC-CMV vector (Invitrogen Corp., San Diego, CA). Stable cell lines were generated by transfecting MDCK cells with the T-Nedd4 or T-ΔC2-Nedd4 constructs using LipofectAMINE (Life Technologies, Inc.) and selection in 1 mg/ml of G418 (Life Technologies, Inc.). Positive clones were tested for protein expression using anti-T7 antibodies (Novagen, Madison, WI).

Immunostaining and Confocal Microscopy—MDCK cells, either wild type or those expressing T-Nedd4 or T-ΔC2-Nedd4, were grown on permeant filters (Falcon 0.4-μm pore size, 6-well format; Becton Dickinson Labware, Lincoln Park, NJ) and then treated (or not) with Ca^{2+} plus ionomycin as described above. Following treatment, filters were washed and fixed briefly in 10% neutral buffered formalin, washed twice in phosphate-buffered saline, and stored at 4 °C. Cells on membranes were then permeabilized at room temperature in a humidity chamber for 30 min with 1% Triton X-100 in Tris-buffered saline (30 mM Tris, pH 7.5, 150 mM NaCl). The filters were cut from the inserts and incubated in 5% normal goat serum in antibody diluting buffer (Dimension Laboratories, Mississauga, ON, Canada) for 30 min followed by incubation for 2 h in the primary antibody (either 10 μg/ml of affinity-pure anti-Nedd4-WWII antibodies or 1:1000 dilution of anti-T7 antibodies). Filters were then washed four times with 30 mM Tris, pH 7.5,
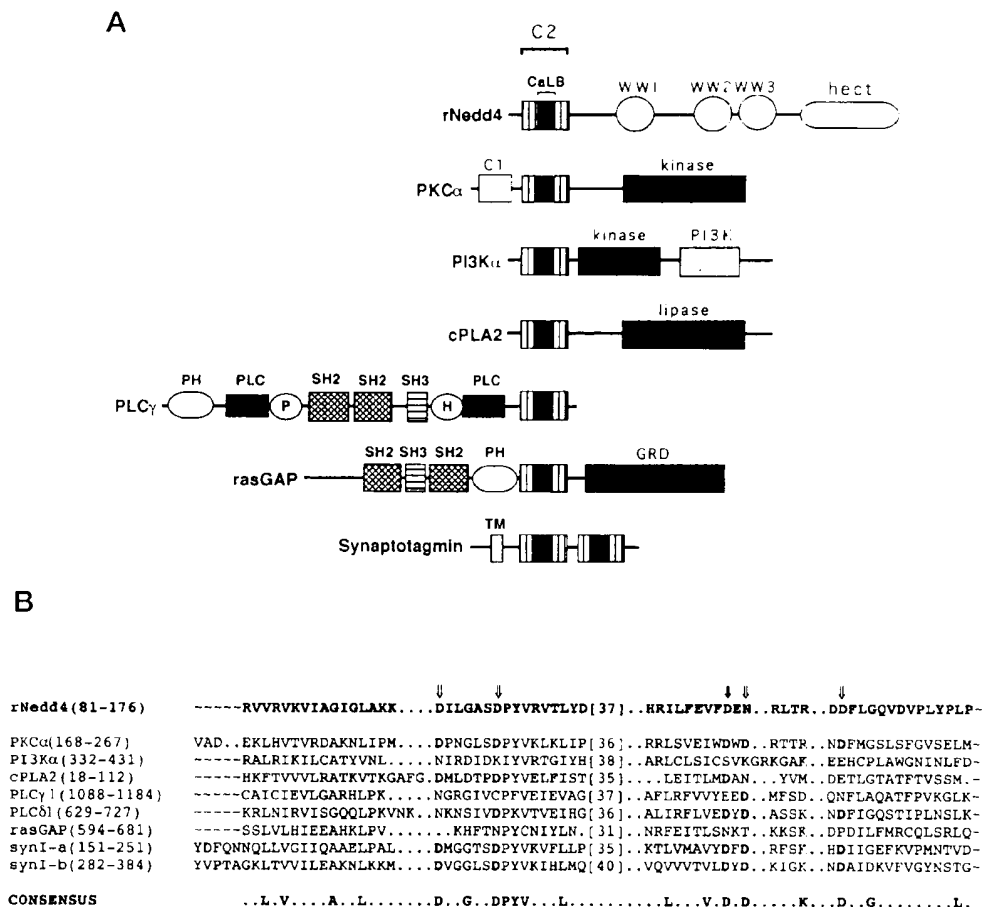
A

B

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rNedd4(81-176)      -----RVVRVKVIAGIGLAKK....DILGASDPYVRVTLYD[37]..HRILFEVFDEN..RLTR..DDFLGQVDVPLYPLP-

PKCα(168-267)       VAD..EKLHVTVRDAKNLIPM....DPNGLSDPYVKLKLIP[36]..RRLSVEIWDWD..RTTR..NDFMGSLSFGVSELM-
PI3Kα(332-431)      -----RALRIKILCATYVNL.....NIRDIDKIYVRTGIYH[38]..ARLCLSICSVKGRKGAF..EEHCPLAWGNINLFD-
cPLA2(18-112)       -----HKFTVVVLRATKVTKGAFG.DMLDTPDPYVELFIST[35]....LEITLMDAN...YVM..DETLGTATFTVSSM.-
PLCγ1(1088-1184)    -----CAICIEVLGARHLPK.....NGRGIVCPFVEIEVAG[37]..AFLRFVVYEED..MFSD..QNFLAQATFPVKGLK-
PLCδ1(629-727)      -----KRLNIRVISGQQLPKVNK..NKNSIVDPKVTVEIHG[36]..ALIRFLVEDYD..ASSK..NDFIGQSTIPLNSLK-
rasGAP(594-681)     -----SSLVLHIEEAHKLPV......KHFTNPYCNIYLN.[31]..NRFEITLSNKT..KKSR..DPDILFMRCQLSRLQ-
synI-a(151-251)     YDFQNNQLLVGIIQAAELPAL....DMGGTSDPYVKVFLLP[35]..KTLVMAVYDFD..RFSF..HDIIGEFKVPMNTVD-
synI-b(282-384)     YVPTAGKLTVVILEAKNLKKM....DVGGLSDPYVKIHLMQ[40]..VQVVVTVLDYD..KIGK..NDAIDKVFVGYNSTG-

CONSENSUS           ..L.V....A..L.......D..G..DPYV...L.........L...V.D.D.....K..D..G........L.
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FIG. 1. **Modular arrangement and sequence alignment of selected C2 domains.** *A*, modular structure of various C2 domain-containing proteins, including rat Nedd4 (*rNedd4*), depicting the C2 domain, 3 WW domains, and a ubiquitin protein ligase (*hect*) domain. *B*, sequence alignment of C2 domains showing the conserved aspartates (*arrows*) involved in coordinating Ca²⁺ in synaptotagmin and phospholipase Cδ. Synaptotagmin is abbreviated *synI*, and the tandem repeats are designated *a* and *b*. The consensus residues at the bottom of the alignment represent residues present in at least 50% of the sequences indicated. *Numbers in brackets* in the sequence indicate the number of intervening residues omitted to optimize the alignment.

150 mM NaCl, 1% bovine serum albumin, 0.05% Triton X-100 (TBS buffer) followed by incubation with biotinylated goat anti-rabbit or anti-mouse secondary antibody (Molecular Probes, Eugene, OR) for 45 min (diluted 1:250 and 1:200 for anti-rabbit and anti-mouse secondary antibodies, respectively). This was followed by several washes in TBS buffer and one final wash in bicarbonate saline buffer (16 mM bicarbonate, 150 mM NaCl, supplemented with 2 mM HEPES, pH 8.5). The filters were then incubated for 45 min with avidin-Texas Red (Molecular Probes) (1:100 dilution in bicarbonate saline) followed by several additional bicarbonate saline washes and finally mounted in Vectashield (Vector Laboratories, Burlingame, CA). The fixed and stained cells were viewed using a Zeiss LSM-4A inverted confocal microscope with ·63 oil objective (NA = 1.3). Texas Red (λ_ex = 596, λ_em = 615) was detected by krypton-argon laser excitation (568 nm) and detection with a photomultiplier after an emission filter (~590 nm). Serial sections were acquired by line averaging the frames (n = 8) at descending z levels. Starting at the apical surface (*i.e.* the coverslip) a minimum of 20 optical sections (each 0.5 μm thick) were obtained for the monolayer. The photomicrographs represent multiple sections in the monolayer observed on each coverslip repeated for at least three independent preparations.

RESULTS

Ca²⁺-dependent Association of Endogenous Nedd4 with Cellular Membranes—Numerous proteins contain regions homologous to the C2 domain (Fig. 1, *A* and *B*), most of which are thought to interact with cellular membranes. To assess whether Nedd4 displays a Ca²⁺-dependent association with

membranes *in vivo*, we examined the changes in the subcellular distribution of endogenously expressed Nedd4 following an increase in intracellular Ca²⁺ in MDCK cells. MDCK cells were used because they represent a well characterized epithelial cell line, forming monolayers of polarized cells when grown to confluency, and because they express high levels of endogenous Nedd4 (2). The elevation in intracellular Ca²⁺ concentrations was achieved by incubating cells with 1 μM ionomycin in the presence of 1 mM Ca²⁺ for 5 min. Cells were then either fractionated and the soluble (S100) or particulate (P100) fractions analyzed for the presence of associated Nedd4 by immunoblotting or immunostained with anti-Nedd4 antibodies to follow intracellular localization of the protein. Fig. 2, *left*, shows that in response to Ca²⁺ plus ionomycin treatment, the majority of endogenous Nedd4 was redistributed from the cytosolic to the particulate fraction. This redistribution was not caused by ionomycin itself, because treatment of cells with ionomycin alone (1 μM) did not alter the primarily cytosolic distribution of Nedd4 (Fig. 2, *right*).

To follow directly the Ca²⁺-dependent subcellular distribution of endogenous Nedd4 in living cells, particularly in cells which are polarized, MDCK cells were grown to confluency on permeant filters to allow them to become polarized. Under these conditions, they form tight junctions and a sheet of high
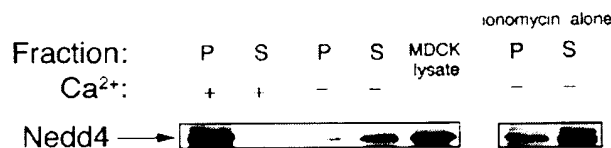
Fraction:		P	S	P	S	MDCK lysate		P	S
Ca²⁺:		+	+	−	−			−	−

Ca²⁺: uses Ca^{2+}

Nedd4 ⟶

Fig. 2. **Ca²⁺-induced association of Nedd4 with crude membranes of MDCK cells.** MDCK cells serum-starved for 16 h were incubated for 5 min at 37 °C in the presence (+) or absence (−) of 1 μM ionomycin plus 1 mM CaCl₂, homogenized, and separated into particulate P100 (*P*) and soluble S100 (*S*) fractions by high speed centrifugation. Nedd4 proteins were then immunoprecipitated from each fraction with anti-Nedd4 antibodies (anti-Nedd4-WW2), separated on 8% SDS-PAGE, and immunoblotted with Nedd4-WW2 antibodies (*left*). A control depicting endogenous expression of Nedd4 in MDCK cells (*MDCK lysate*) is also included (*left*). MDCK cells treated with ionomycin alone exhibited the same Nedd4 distribution profile as the untreated cells (*right*).

resistance epithelial monolayer.[2] Following treatment of cells without or with Ca^{2+} plus ionomycin, cells were permeabilized and immunostained with anti-Nedd4 antibodies followed by Texas Red-conjugated secondary antibodies, as described under "Experimental Procedures." The cells were then viewed by confocal microscopy using a LSM-410 Zeiss laser scanning microscope. Fig. 3, *A* and *B*, depicts two series of horizontal (XY) sections of MDCK cells stained with affinity-pure anti-Nedd4 antibodies taken at 0.5-μm intervals starting from the apical (top) surface and representing either untreated control cells (Fig. 3*B*) or Ca^{2+} plus ionomycin-treated cells (Fig. 3*A*). As evident from Fig. 3*A*, after Ca^{2+} plus ionomycin treatment, the majority of Nedd4 was localized to the plasma membrane, preferentially accumulating at the apical and lateral membranes; only base-line levels (~5%) of Nedd4 were detected in the membrane fraction of untreated cells (Fig. 3*B*). Accordingly, vertical (XZ) reconstructions (summations) of all the images depicted in Fig. 3, *A* and *B*, indeed demonstrated strong staining for Nedd4 at the apical (top) region of the treated cells (Fig. 3*C*), whereas the distribution of Nedd4 in the untreated cells was relatively even throughout the cell (Fig. 3*D*). A time course of Nedd4 association with the apical/subapical membrane revealed the interaction persisted for approximately 30–45 min (data not shown). These results, therefore, support the above observation of Ca^{2+}-induced association of Nedd4 with membranes. Moreover, they demonstrate that in polarized epithelia such as MDCK cells, endogenously expressed Nedd4 is preferentially redistributed to the apical and lateral regions of the cell in response to elevated intracellular levels of Ca^{2+}.

The Nedd4-C2 Domain Is Sufficient to Mediate Ca²⁺-dependent Membrane Association in Vitro—To determine whether the Ca^{2+}-dependent membrane association of Nedd4 was due to the action of the C2 domain alone and not to other regions of the protein, a GST fusion protein encompassing the C2 domain was constructed and its association with purified membranes in response to Ca^{2+} was determined. Thus, 200 μg of MDCK crude membrane fractions (P100) were incubated with soluble GST-Nedd4-C2 or GST alone (10 nM each) in the presence of increasing concentrations of Ca^{2+} (+1 mM EGTA). Membranes were then collected and proteins separated on SDS-PAGE and immunoblotted with anti-GST antibodies to determine the amount of membrane-associated GST-Nedd4-C2. Our results show (Fig. 4) a basal association between the P100 fraction and the GST-Nedd4-C2 in the range of 0–300 nM Ca^{2+}, consistent with our initial observation of limited membrane association of Nedd4 even in the absence of elevated intracellular Ca^{2+} levels (Fig. 2*A*). However, there was a clear and reproducible increase

in membrane-associated GST-Nedd4-C2 when Ca^{2+} concentrations were raised from 300 to 500 nM, with no further increase in binding at concentrations up to 1000 nM (Fig. 4). GST alone did not bind to MDCK membranes. These results therefore demonstrate that the C2 domain of Nedd4 alone was sufficient to bind MDCK membranes *in vitro* and that this binding was Ca^{2+}-dependent at Ca^{2+} concentrations ~300 nM.

In several Ca^{2+}-responsive proteins, the C2 domain displays specificity toward negatively charged phospholipids (22, 30, 46). To determine whether the C2 domain of Nedd4 binds phospholipids and whether it shows preference toward negatively charged ones, binding experiments with purified GST-Nedd4-C2 and purified liposomes of varying composition were performed. Phosphatidylserine, phosphatidylinositol stabilized with phosphatidylcholine, and pure phosphatidylcholine were prepared as described under "Experimental Procedures." They were then incubated with soluble GST-Nedd4-C2 or GST alone (10 nM each) in the absence or presence of 1 mM Ca^{2+}. Liposomes were subsequently sedimented and associated GST-Nedd4-C2 (or GST alone) analyzed by immunoblotting with anti-GST antibodies as described above. Fig. 5 shows that GST-Nedd4-C2, but not GST alone, was able to bind to pure phospholipid liposomes. This association was partially (~50%) augmented in the presence of Ca^{2+} but did not display obvious specificity for any lipid type, because no significant difference between the association of the GST-Nedd4-C2 with pure phosphatidylcholine, pure phosphatidylserine, or a 1:1 mixture of phosphatidylcholine/phosphatidylinositol was detected. Thus, the C2 domain of Nedd4 alone is capable of binding in a Ca^{2+}-responsive manner to either MDCK membranes or to pure phospholipids.

Deletion of the C2 Domain Abrogates Ca²⁺-dependent Membrane Association of Nedd4 in Vivo—To test whether the Nedd4-C2 domain was responsible for the observed Ca^{2+}-dependent membrane association of Nedd4 *in vivo*, stable MDCK cell lines expressing either T7 epitope-tagged full-length Nedd4 (T-Nedd4) or T7 epitope-tagged C2-deleted Nedd4 (T-ΔC2-Nedd4) were generated (Fig. 6*A*). Several clones of transfected MDCK cells were tested for protein production, and the two clones expressing approximately equal amounts of protein, T-Nedd4 (clone 2–11) and T-ΔC2-Nedd4 (clone 2–27) (Fig. 6*B*), were then tested for Ca^{2+}-dependent subcellular localization using immunostaining with anti-T7 antibodies and confocal microscopy. To ensure that the addition of the T7 epitope onto Nedd4 by itself did not affect membrane localization in response to calcium, T-Nedd4 (2–11) monolayers grown on filters were treated with Ca^{2+}/ionomycin as above, fixed, stained with anti-T7 antibodies, and examined with the confocal microscope. Our results show that the T-Nedd4 heterologously expressed in MDCK cells displayed a similar Ca^{2+} response as the endogenous Nedd4 (Fig. 3) in that it was mainly cytosolic in untreated cells but was localized to the plasma membrane in response to Ca^{2+} plus ionomycin treatment (Fig. 6*C*). We therefore proceeded to test membrane localization of the C2-deleted (T-ΔC2-Nedd4) protein in MDCK cells following Ca^{2+}/ionomycin treatment using fluorescence microscopy. In contrast to the Ca^{2+}-induced membrane association seen with T-Nedd4 construct (Fig. 7*b*), the T-ΔC2-Nedd4 did not associate with the plasma membrane following Ca^{2+} plus ionomycin treatment (Fig. 7*d*). As before, no significant plasma membrane association of Nedd4 was observed in untreated control cells (Fig. 7, *a* and *c*). Although the anti-T7 antibody displays a nonspecific cross-reactive nuclear staining, this was unrelated to the expression of transfected Nedd4, because there was no cytosolic or membrane staining in untransfected MDCK cells (Fig. 7*e*).

Taken together, these results indicate that the C2 domain of

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[2] S. Dho and D. Rotin, unpublished data.

A

apical

20 um   basolateral

B

apical

20 um   basolateral

C   + Ca²⁺

D   - Ca²⁺
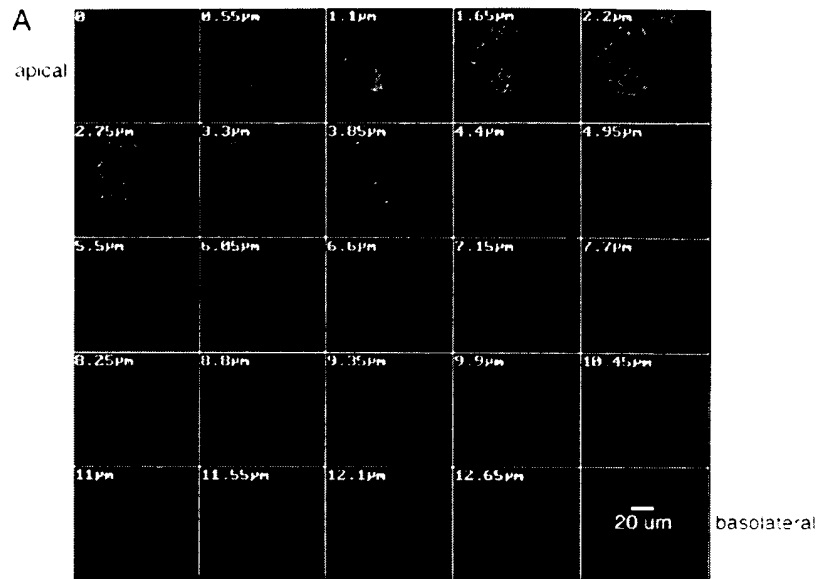
FIG. 3. **Confocal micrographs of Ca²⁺-dependent apical and lateral membrane localization of Nedd4 in polarized MDCK cells.** MDCK cells were grown to confluency on permeable filters to allow them to become polarized. They were then either not treated ($-Ca^{2+}$) or treated ($+Ca^{2+}$) with Ca²⁺ plus ionomycin, as described in Fig. 2. Cells were subsequently fixed with 10% formalin and immunostained with affinity-pure anti-rabbit Nedd4 primary antibodies followed by goat anti-rabbit Texas Red conjugated secondary antibodies. Serial cross-sections (XY) of polarized MDCK cells either Ca²⁺ treated (*A*) or untreated (*B*) were taken from the top of the monolayer (apical surface) down to the glass slide (basal surface) at 0.5-μm intervals. These XY images were then summed to generate vertical (XZ) image reconstructions depicting Nedd4 vertical distribution in Ca²⁺-treated (*C*) and untreated (*D*) polarized MDCK cells.

[Ca²⁺] (nM)
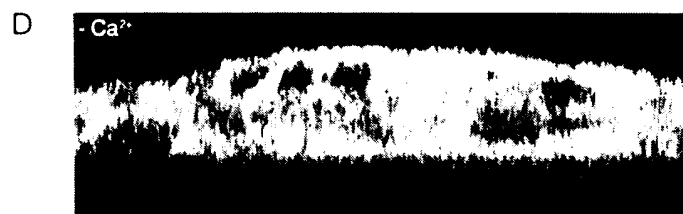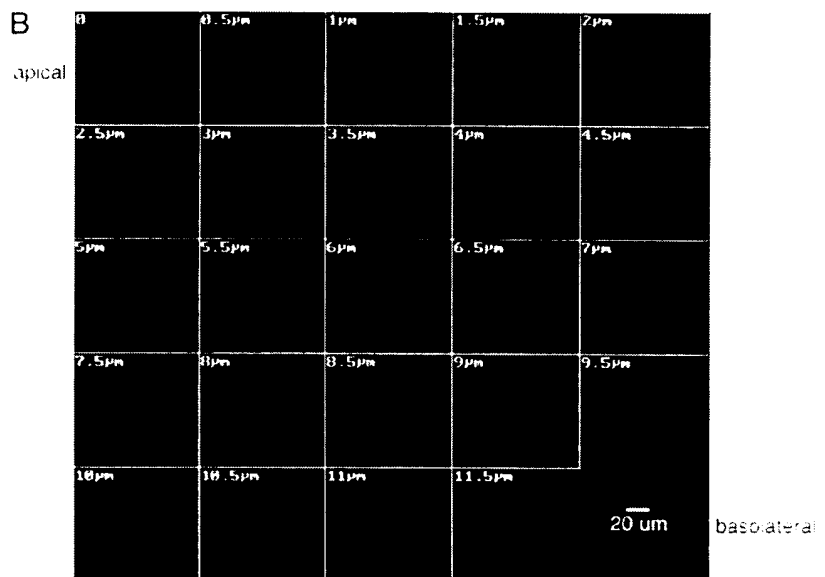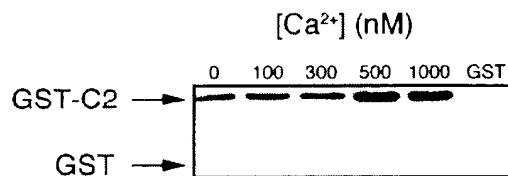
FIG. 4. **Ca²⁺-dependent binding of GST-Nedd4-C2 to MDCK crude membranes.** Two hundred micrograms of crude MDCK membranes were incubated with GST alone or with GST-Nedd4-C2 (10 nM each) in the presence of the indicated concentrations of Ca²⁺ for 30 min at 30 °C. The membranes were collected and proteins separated on 10% SDS-PAGE and transferred to nitrocellulose and immunoblotted with anti-GST antibodies. *Arrows* indicate the GST-C2 fusion protein.
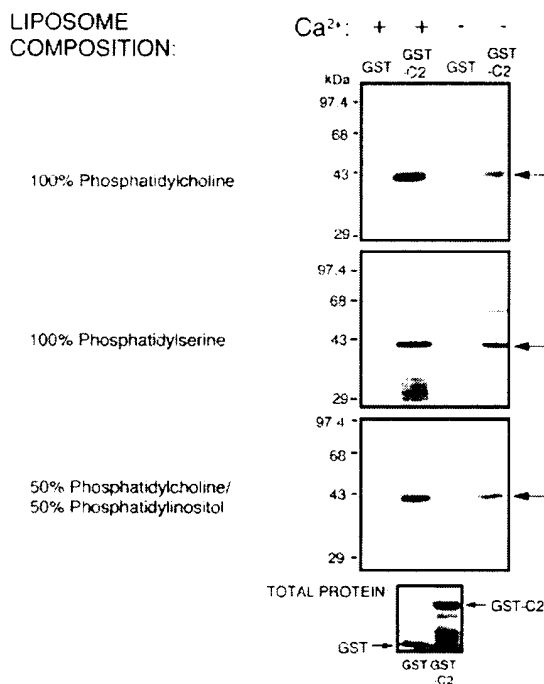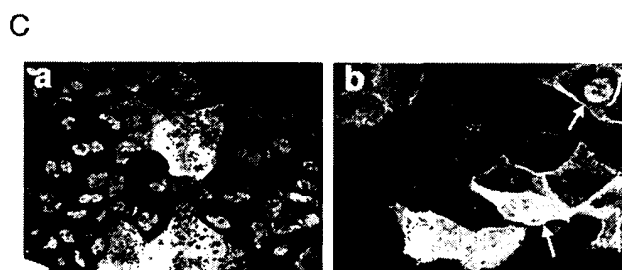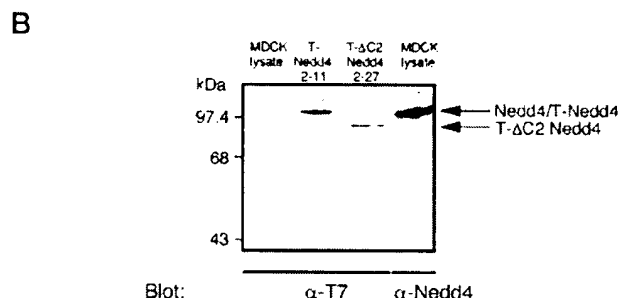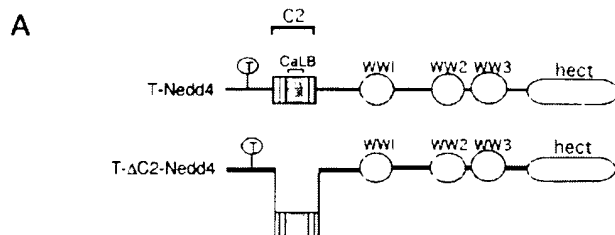

FIG. 5. **Ca²⁺-dependent association of GST-Nedd4-C2 with phospholipids.** Liposomes of the indicated composition were incubated with 10 nM purified GST or GST-Nedd4-C2 in the presence of 1 mM Ca²⁺. Liposomes were then sedimented and the associated protein separated on 10% SDS-PAGE and immunoblotted with anti-GST antibodies (as described in Fig. 4). The *bottom panel* represents the total amount of protein used in the binding experiments.

A

B

C

FIG. 6. **Epitope-tagged Nedd4 in transfected MDCK cells displays Ca²⁺-dependent plasma membrane association.** *A*, schematic representation of the T7 epitope-tagged Nedd4 (*T-Nedd4*) and the T7 epitope-tagged C2-deleted Nedd4 (*T-ΔC2-Nedd4*) constructs used for stable transfection into MDCK cells. *B*, expression of T-Nedd4 (clone 2–11) and T-ΔC2-Nedd4 (clone 2–27) in MDCK cells stably transfected with the constructs depicted in *A*. Proteins from the lysate of the transfected cells were separated on 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with monoclonal anti-T7 antibodies to determine levels of protein expression. Lysate of untransfected MDCK cells (*MDCK lysate*) immunoblotted with affinity-pure anti-Nedd4-WWII antibodies used as a control is depicted in the *right lane*. *C*, intracellular localization of T-Nedd4 in untreated (*a*) or Ca²⁺ plus ionomycin-treated (*b*) transfected MDCK cells as viewed with the confocal microscope. Cells were fixed and permeabilized as described in Fig. 3 and immunostained with monoclonal anti-T7 antibodies followed by goat anti-mouse secondary antibodies conjugated to Texas Red. XY images shown represent slices through the subapical region of the membrane taken 3 μm from the apical surface.

Nedd4 is necessary for the Ca²⁺-induced redistribution of the protein from the cytosol to the plasma membrane.

DISCUSSION

In this report, we show that the ubiquitin protein ligase Nedd4 possesses a functional C2 domain; upon increases in cytosolic calcium, endogenous Nedd4 associates with membranes and, interestingly, displays polarized distribution in that it localizes to the apical and lateral membranes of polarized MDCK cells. This membrane localization is abolished in the absence of the C2 domain. Moreover, the C2 domain alone is sufficient to mediate Ca²⁺-dependent membrane and phospholipid association.

Several mechanisms could explain the localization of Nedd4 to the apical region of MDCK cells polarized epithelia. The distribution of various lipid classes in the membrane regions of cultured MDCK cells has been studied extensively (47). These reports demonstrate that the apical membrane has a high glycosphingolipid content. This high glycosphingolipid content at the apical membrane may serve to increase the phospholipid packing density at this region, thereby providing a favorable environment for Nedd4-C2 association. Clearly, a greater understanding of the dynamics of C2-lipid interactions is necessary to fully understand the translocation mechanism. Alternatively, other factors may be involved in binding of Nedd4 to the apical site. Although in this report we show that a Nedd4-C2 fusion protein is sufficient to bind purified membrane in a Ca²⁺-dependent manner, this does not exclude the involvement of other proteins that are either translocalized to the apical membrane upon increases in cellular calcium or that are already present there and retain Nedd4 upon its Ca²⁺-dependent (C2-mediated) arrival. Indeed, Nedd4 possesses three WW domains that are known to be involved in mediating protein-protein interactions (2, 9). Thus, as documented for
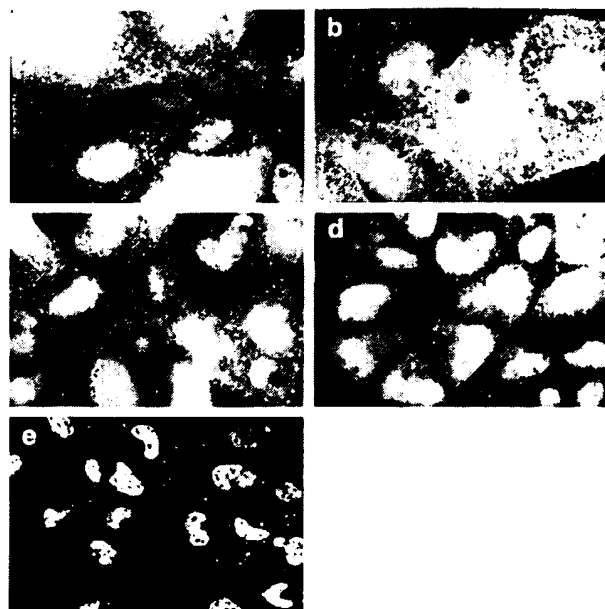
FIG. 7. **Lack of Ca²⁺-induced membrane localization of C2-deleted Nedd4 stably expressed in MDCK cells.** Fluorescent micrographs of intracellular localization of T-Nedd4 (*a* and *b*) and T-ΔC2-Nedd4 (*c* and *d*) in untreated (*a* and *c*), in Ca²⁺ plus ionomycin-treated (*b* and *d*) transfected MDCK cells, or in untransfected cells (*e*). Cells were fixed and permeabilized as described in Fig. 3 and immunostained with monoclonal anti-T7 antibodies followed by goat anti-mouse secondary antibodies conjugated to Texas Red. Staining with anti-T7 antibodies gave a background nuclear stain which was unrelated to transfection (*e*). Photographs represent total fluorescence as viewed with rhodamine optics on a standard fluorescent microscope. *Arrows* indicate areas of plasma membrane localization.

other C2 domains, it is possible that C2-protein interactions may be involved in Nedd4 cellular translocation as well.

The Ca²⁺-dependent polarized membrane localization of Nedd4 in epithelial cells is reminiscent of the specialized translocation of synaptotagmin to nerve terminals in response to calcium. Synaptotagmin is an essential component of the Ca²⁺ sensor for the final step in nerve terminal fusion leading to neurotransmitter release (for review, see Ref. 37) (48). The function of synaptotagmin in exocytosis is mediated by its association with syntaxins (32), which are plasma membrane proteins that form complexes with other vesicular proteins (49, 50). This interaction serves to localize synaptic vesicles to the nerve terminal for proper transmitter release. Although the function of the Nedd4 apical/lateral membrane translocation is not known, it is possible that its calcium responsive targeting may increase the specificity of interactions of this ubiquitin protein ligase with its substrate(s); in this model, the apically located substrate would only become ubiquitinated following an increase in intracellular levels of Ca²⁺.

The interaction between the C2 domain and its calcium ligand is thought to occur via 5 conserved aspartates that line the opening of the C2 key motif, corresponding to Asp-172, -178, -230, -232, -238 of synaptotagmin (25–27). Many of the C2 domains identified thus far including that of Nedd4 (Fig. 1*B*), however, do not possess the full complement of conserved aspartates (11, 12). Although available structural data suggests that all five aspartates are utilized for calcium binding (25, 26), the C2 domains of molecules such as rasGAP and cytosolic phospholipase A₂, which have been shown to mediate calcium-dependent membrane localization, do not possess all these conserved aspartates (18, 22); in fact, the 40-amino acid CaLB region of rasGAP is sufficient to mediate calcium-dependent

phospholipid binding (22, 51), despite the fact that it does not possess any of the conserved aspartates. In these molecules the aspartate residues are often replaced with other oxygen-containing side chains (Asn, Thr) that may be able to coordinate Ca²⁺ binding.[3] Thus, the observation of C2-dependent association of Nedd4 with the plasma membrane, despite lacking one of its conserved aspartates, is consistent with findings in other C2 domains. Whether the differences in conservation of coordinating residues reflect the different affinities for calcium is presently unclear.

The Ca²⁺-dependent membrane and phospholipid binding properties of Nedd4 C2 are similar to what has been reported for other C2 domains. There was a visible increase in binding of purified GST-C2 Nedd4 to cellular membranes between 300 and 500 μM Ca²⁺. This finding is consistent with similar binding studies done with rasGAP (51), where GST-GAP binding was exponential between 10⁻⁷ and 10⁻⁶ M free Ca²⁺. In addition, previous studies with the C2 domains of synaptotagmin and rasGAP (22, 30, 46) show a preference of the C2 domain for negatively charged phospholipids upon Ca²⁺ binding. Contrary to this, our results show lack of preference of Nedd4-C2 domain toward negatively charged phospholipids. This finding, however, is in agreement with that of the cytosolic phospholipase A₂ C2 domain, which was also shown not to have such preference (23); in fact, that domain shows approximately 10-fold higher affinity for zwitterionic (phosphatidylcholine) phospholipids than anionic phospholipids.[3]

We have recently demonstrated association of Nedd4 with ENaC (2). We have also shown that ENaC is ubiquitinated *in vivo* (52). Although we do not know yet whether Nedd4 is directly involved in ubiquitinating ENaC, this is a likely possibility. ENaC is located at the apical membrane of epithelial cells (such as those in the distal nephron, distal colon, and lung epithelia), and one possible scenario is that elevation of intracellular Ca²⁺ may target Nedd4 to the apical membrane where ENaC is located. This would then allow for the Nedd4-WW domains to associate with the channel and for the Nedd4-hect domain to ubiquitinate it. This model therefore predicts that elevation of intracellular Ca²⁺ would cause inhibition of ENaC activity resulting from enhanced channel ubiquitination and degradation. Indeed, earlier studies have documented inhibition of amiloride-sensitive Na⁺ channel activity by elevated intracellular Ca²⁺ levels (53). Moreover, our own recent studies with ENaC expressed in MDCK cells revealed a biphasic inhibition of ENaC activity following dialysis with 1 μM free Ca²⁺, an initial rapid (within 5 min) inhibition probably caused by changes in channel gating, followed by a secondary slow inhibition (5–20 min), which we suspect may originate from a decrease in channel numbers at the cell surface.[4] We speculate that this slow phase may involve Nedd4 activity. This model does not preclude the possibility that the Nedd4-C2 domain is also involved in ENaC endocytosis/degradation, perhaps in an analogy to the second C2 domain of synaptotagmin, which mediates interactions with the clathrin-associated protein AP2 (38).

In summary, the presence of a functional C2 domain in Nedd4 may help in determining the array of substrates (ENaC and others) which are targeted for ubiquitination by this ubiquitin protein ligase by directing subcellular distribution of Nedd4 to the location of these proteins in a Ca²⁺-dependent fashion. Specificity of binding to these putative target protein(s) may be then mediated by the WW domains and subsequent ubiquitination by the hect domain.

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[3] J. Falke, personal communication.
[4] T. Ishikawa, Y. Marunaka, and D. Rotin, unpublished observations.

Characterization of Nedd4-C2 Domain

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